

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
7 October 2004 (07.10.2004)

PCT

(10) International Publication Number
WO 2004/084831 A2

- (51) International Patent Classification⁷: **A61K**
- (21) International Application Number: **PCT/US2004/008677**
- (22) International Filing Date: 19 March 2004 (19.03.2004)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/457,172 24 March 2003 (24.03.2003) US
- (71) Applicant (for all designated States except US): **MERCK & CO. INC.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **JANSEN, Kathrin**, U. [DI/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **SCHULTZ, Loren, D.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **NEEPER, Michael, P.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **MARKUS, Henry, Z.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).
- (74) Common Representative: **MERCK & CO. INC.**; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— without international search report and to be republished upon receipt of that report

(Continued on next page)

(54) Title: OPTIMIZED EXPRESSION OF HPV 31 L1 IN YEAST

HPV31 L1 total rebuild nucleotide and amino acid sequence:

```

1  H S L W R P S E A T V Y L P P V P
   ATGTCTTTGT GGAGCATCAG TGAGCTACAG GTCTACTTGG CACGAGTCCC
51  V S K V V S T D E Y V T R T W I Y
   AGTCTCTAG GTGTCTCTCA CCAGCAATA GGTCAACAGA ACGAATCTCT
91  Y H A G S A R L L T V G H P Y Y
   ACTACAGGCG TGTTTCTGCT AGATTCTTGA CCGTGGTCA CCGATCTAC
131 S I P K S D N P K K I V Y P K V S
   TCTATCCGAA AGTCTGACAA CCGAAGAGAG ATGTGTGTC CAAAGTCTTC
171 G L O V B Y F R V R L P D P N K F
   TGTTTGGCAA TACAGATGCT TCGAGTGGAG ATTCGAGAG CCAACAGTGT
211 G F P D T S F Y H P E T Q R L Y
   TCGTTTTCGC AGACAGCTCT TTCTACAGCC CAGAACCCA AAGATTGTCT
251 W A C V G L E V R G O P L G V G
   TGGTCTGTGT TCGTTTGGAA AGTGTGTAGA GTGACCAAT TGTGTGTGG
291 I S G H P L L R K F D D T E N S N
   TATCTCTGCT CACCCATGTT TGACCAAGTT CAGAGACACC GAAACTCTCA
331 R Y A G G P G T D W R E C I S N
   ACAGATAGCG TGTTGTGTCA GTTACAGACA ACGAGATG TGTTCTATG
371 D Y K T Q L C L L G C K P P I G
   GACTACAGCG AAGCAATATT GTTGTGTGTT GTTGTGTAGC CACCAATCGG
411 E H W G K S P C S V V A I T P G
   TGAGACTGGG GTTAAGGTTT CTCTATGTTT TACAGAGCT ATCAACCCAG
451 D C P L E L K N S V I Q D G D
   GTGACTGTCC ACCATTGGAA TTGAGAACT CTGTATCA GAAGCGTAC
491 M V D T G F G A N D F T A L Q O T
   ATGTGTGACA CCGTGTTCGG TGCTATGGAC TTCAACGCTI TCGAAGACAC
531 K S H V P L D I C H S I C K Y P D
   CAGTCTTACG GTCCCATGCG ACATCTGTAA CTCTATCTGT AAGTACCCAG
571 Y L K M V A E P Y G D T L F F Y
   ACTACTTGAH GATGTGTGCT GAACATAGAG GCGACAGCTT GTTCTTCTAC
611 L R R E Q H F V R H F F M R S G T
   TTGTGTAGAG AACAGATGTT GGTAAAGGAC TTCTTCAACA GATTCGGGAC
651 V G E S V P T D L Y I K G S G S T
   GGTAGTGGAA TCTGTGCCAA CCGACCTGTA CATCAAGGCG TCGGTGTCCA
691 A T L A H S T Y F P T P S G S N
   CCGTACCTCT GGTAACTGCG ACTTACTTCC CACTCATGTC TGCTGTCTAG
731 V T S D A O I F M K P Y W N Q R A
   GTACCTGCGG AGCTTCABAT CTTCAGCAAG CCACTACTGA TCGAGCTGCG
771 Q G H M N G T C V G N O L F V T V
   ACAGGTCAC AACAGGTTA TCTGTGGGGA TACCAAGCTG TTGTGTAGCT
811 V D T T R S T H N S V C A A I A
   TGTTGTATAC CAGCGTGTCT ACCAAGATGT CTGTCTGTGCG TCGAATGCTG
851 H S D T T F K S S W F A E Y L R H
   AACTGTGACA CTACTCTCAA GTCTCTAAC TTCAAGAGTT AACTGTAGACA
891 G E E F D L Q F I F O L C K I T L
   TGTTGAGGAA TTGATGTGTC AATTCACTCT CAGTGTGTGCG AAGTACCCCG
931 S A D I N T Y I H S H N P A I L
   TTGTGTGTGA CATCATGAGC TACATGACA GTATGAGCCG TGCACTGTG
971 E D W N F G L T T P S G S L E D
   GAGGACTGGA ACTTGCTGCT GAGCACTECA CTTCTCGGTT CTTTGAGAGA

```

(57) Abstract: Synthetic DNA molecules encoding the HPV31 L1 protein are provided. Specifically, the present invention provides polynucleotides encoding HPV31 L1 protein, wherein said polynucleotides are free from internal transcription termination signals that are recognized by yeast. Also provided are synthetic polynucleotides encoding HPV31 L1 wherein the polynucleotides have been codon-optimized for high level expression in a yeast cell. The synthetic molecules may be used to produce HPV31 virus-like particles (VLPs), and to produce vaccines and pharmaceutical compositions comprising the HPV31 VLPs. The vaccines of the present invention provide effective immunoprophylaxis against papillomavirus infection through neutralizing antibody and cell-mediated immunity.

21561
#8



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TITLE OF THE INVENTION
OPTIMIZED EXPRESSION OF HPV 31 L1 IN YEAST

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 This application claims the benefit of U.S. Provisional Application No. 60/457,172 filed March 24, 2003, the contents of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

- 10 The present invention relates generally to the therapy of human papillomavirus (HPV). More specifically, the present invention relates to synthetic polynucleotides encoding HPV31 L1 protein, and to recombinant vectors and hosts comprising said polynucleotides. This invention also relates to HPV31 virus-like particles (VLPs) and to their use in vaccines and pharmaceutical compositions for preventing and treating HPV.

15 BACKGROUND OF THE INVENTION

- There are more than 80 types of human papillomavirus (HPV), many of which have been associated with a wide variety of biological phenotypes, from benign proliferative warts to malignant carcinomas (for review, *see* McMurray et al., *Int. J. Exp. Pathol.* 82(1): 15-33 (2001)). HPV6 and HPV11 are the types most commonly associated with benign warts, nonmalignant condylomata
20 acuminate and/or low-grade dysplasia of the genital or respiratory mucosa. HPV16 and HPV18 are the high-risk types most frequently associated with *in situ* and invasive carcinomas of the cervix, vagina, vulva and anal canal. More than 90% of cervical carcinomas are associated with infections of HPV16, HPV18 or the less prevalent oncogenic types HPV31, -33, -45, -52 and -58 (Schiffman et al., *J. Natl. Cancer Inst.* 85(12): 958-64 (1993)). The observation that HPV DNA is detected in 90-100% of cervical
25 cancers provides strong epidemiological evidence that HPVs cause cervical carcinoma (*see* Bosch et al., *J. Clin. Pathol.* 55: 244-265 (2002)).

- Papillomaviruses are small (50-60 nm), nonenveloped, icosahedral DNA viruses that encode up to eight early and two late genes. The open reading frames (ORFs) of the viral genomes are designated E1 to E7, and L1 and L2, where "E" denotes early and "L" denotes late. L1 and L2 code for
30 virus capsid proteins, while the E genes are associated with functions such as viral replication and cellular transformation.

 The L1 protein is the major capsid protein and has a molecular weight of 55-60 kDa. The L2 protein is a minor capsid protein. Immunological data suggest that most of the L2 protein is internal to the L1 protein. Both the L1 and L2 proteins are highly conserved among different papillomaviruses.

Expression of the L1 protein or a combination of the L1 and L2 proteins in yeast, insect cells, mammalian cells or bacteria leads to self-assembly of virus-like particles (VLPs) (for review, *see* Schiller and Roden, in *Papillomavirus Reviews: Current Research on Papillomaviruses*; Lacey, ed. Leeds, UK: Leeds Medical Information, pp 101-12 (1996)). VLPs are morphologically similar to authentic virions and are capable of inducing high titers of neutralizing antibodies upon administration into an animal or a human. Because VLPs do not contain the potentially oncogenic viral genome, they present a safe alternative to use of live virus in HPV vaccine development (for review, *see* Schiller and Hidesheim, *J. Clin. Virol.* 19: 67-74 (2000)). For this reason, the L1 and L2 genes have been identified as immunological targets for the development of prophylactic and therapeutic vaccines for HPV infection and disease.

HPV vaccine development and commercialization have been hindered by difficulties associated with obtaining high expression levels of capsid proteins in successfully transformed host organisms, limiting the production of purified protein. Therefore, despite the identification of wild-type nucleotide sequences encoding HPV L1 proteins such as HPV31 L1 proteins (Goldsborough et al., *Virology* 171(1): 306-311 (1989), it would be highly desirable to develop a readily renewable source of crude HPV proteins that utilizes HPV31 L1-encoding nucleotide sequences that are optimized for expression in the intended host cell. Additionally, it would be useful to produce large quantities of HPV31 L1 VLPs having the immunity-conferring properties of the native proteins for use in vaccine development.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods to elicit or enhance immunity to the protein products expressed by HPV31 L1 genes, which have been associated with cervical cancer. Specifically, the present invention provides polynucleotides encoding HPV31 L1 protein, wherein said polynucleotides are free from internal transcription termination signals that are recognized by yeast. Also provided are synthetic polynucleotides encoding HPV31 L1 wherein the polynucleotides have been codon-optimized for high level expression in a yeast cell. The present invention further provides HPV31 virus-like particles (VLPs) and discloses use of said VLPs in immunogenic compositions and vaccines for the prevention and/or treatment of HPV disease or HPV-associated cancer.

The present invention relates to synthetic DNA molecules encoding the HPV31 L1 protein. In one aspect of the invention, the nucleotide sequence of the synthetic molecule is altered to eliminate transcription termination signals that are recognized by yeast. In another aspect, the codons of the synthetic molecules are designed so as to use the codons preferred by a yeast cell. The synthetic

molecules may be used as a source of HPV31 L1 protein, which may self-assemble into VLPs. Said VLPs may be used in a VLP-based vaccine.

A particular embodiment of the present invention comprises a synthetic nucleic acid molecule which encodes the HPV31 L1 protein as set forth in SEQ ID NO:4, said nucleic acid molecule comprising a sequence of nucleotides as set forth in SEQ ID NO:2 or SEQ ID NO:3.

As stated above, provided herein are synthetic polynucleotides encoding the HPV31 L1 gene which are free from transcription termination signals that are recognized by yeast. This invention also provides synthetic polynucleotides encoding HPV 31 L1 as described, which are further altered so as to contain codons that are preferred by yeast cells.

Also provided are recombinant vectors and recombinant host cells, both prokaryotic and eukaryotic, which contain the nucleic acid molecules disclosed throughout this specification.

The present invention relates to a process for expressing an HPV31 L1 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid encoding an HPV31 L1 protein into a yeast host cell; wherein the nucleic acid molecule is free from internal transcription termination signals that are recognized by yeast and; (b) culturing the yeast host cell under conditions which allow expression of said HPV31 L1 protein.

The present invention further relates to a process for expressing an HPV31 L1 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid encoding an HPV31 L1 protein into a yeast host cell; wherein the nucleic acid molecule is codon-optimized for optimal expression in the yeast host cell and; (b) culturing the yeast host cell under conditions which allow expression of said HPV31 L1 protein.

In preferred embodiments, the nucleic acid comprises a sequence of nucleotides as set forth in SEQ ID NO:2 or SEQ ID NO:3.

This invention also relates to HPV31 virus-like particles (VLPs), methods of producing HPV31 VLPs, and methods of using HPV31 VLPs.

In a preferred embodiment of the invention, the HPV31 VLPs are produced in yeast. In a further preferred embodiment, the yeast is selected from the group consisting of: *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces fragilis*, *Kluveromyces lactis*, and *Schizosaccharomyces pombe*.

Another aspect of this invention is an HPV31 VLP, which comprises an HPV31 L1 protein produced by a HPV31 L1 gene which is free from transcription termination signals that are recognized by yeast.

Yet another aspect of this invention is an HPV31 VLP, which comprises an HPV31 L1 protein produced by a codon-optimized HPV31 L1 gene. In a preferred embodiment of this aspect of the

invention, the codon-optimized HPV31 L1 gene consists essentially of a sequence of nucleotides as set forth in SEQ ID NO:2 or SEQ ID NO:3.

This invention also provides a method for inducing an immune response in an animal comprising administering HPV31 virus-like particles to the animal. In a preferred embodiment, the HPV31 VLPs are produced by a codon-optimized gene. In a further preferred embodiment, the HPV31 VLPs are produced by a gene that is free from transcription termination sequences that are recognized by yeast.

Yet another aspect of this invention is a method of preventing or treating HPV-associated cervical cancer comprising administering to a mammal a vaccine comprising HPV31 VLPs. In a preferred embodiment of this aspect of the invention, the HPV31 VLPs are produced in yeast.

This invention also relates to a vaccine comprising HPV31 virus-like particles (VLPs).

In an alternative embodiment of this aspect of the invention, the vaccine further comprises VLPs of at least one additional HPV type. In a preferred embodiment, the at least one additional HPV type is selected from the group consisting of: HPV6, HPV11, HPV16, HPV18, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV55, HPV56, HPV58, HPV59, and HPV68.

This invention also relates to pharmaceutical compositions comprising HPV 31 virus-like particles. Further, this invention relates to pharmaceutical compositions comprising HPV31 VLPs and VLPs of at least one additional HPV type. In a preferred embodiment, the at least one additional HPV type is selected from the group consisting of: HPV6, HPV11, HPV16, HPV18, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV55, HPV56, HPV58, HPV59, and HPV68.

As used throughout the specification and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

As used throughout the specification and appended claims, the following definitions and abbreviations apply:

The term "promoter" refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or "upstream activating sequences" or inhibiting sequences termed "silencers".

The term "vector" refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, virus (including adenovirus), bacteriophages and cosmids.

The designation "31 L1 wild-type sequence" refers to the HPV31 L1 sequence disclosed herein as SEQ ID NO:1. Although the HPV 31 L1 wild-type sequence has been described previously, it

is not uncommon to find minor sequence variations between DNAs obtained from clinical isolates. Therefore, a representative HPV31 L1 wild-type sequence was isolated from clinical samples previously shown to contain HPV 31 DNA (see EXAMPLE 1). The 31 L1 wild-type sequence was used as a reference sequence to compare the codon-optimized HPV 31 L1 sequences disclosed herein (see FIGURE 1).

The designation "31 L1 partial rebuild" refers to a construct, disclosed herein (SEQ ID NO:2), in which the HPV31 L1 nucleotide sequence was partially rebuilt to contain yeast-preferred codons for optimal expression in yeast. The 31 L1 partial rebuild comprises alterations in the middle portion of the HPV 31 L1 wild-type nucleotide sequence (nucleotides 697-1249). The complete HPV 31 L1 sequence was also rebuilt with yeast-preferred codons, which is referred to herein as the "31 L1 total rebuild" (SEQ ID NO:3).

The term "effective amount" means sufficient vaccine composition is introduced to produce the adequate levels of the polypeptide, so that an immune response results. One skilled in the art recognizes that this level may vary.

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid for aspartic acid).

The term "mammalian" refers to any mammal, including a human being.

"VLP" or "VLPs" mean(s) virus-like particle or virus-like particles.

"Synthetic" means that the HPV31 L1 gene has been modified so that it contains a sequence of nucleotides that is not the same as the sequence of nucleotides present in the naturally occurring wild-type HPV31 L1 gene. As stated above, synthetic molecules are provided herein comprising a sequence of nucleotides that are altered to eliminate transcription termination signals recognized by yeast. Also provided herein are synthetic molecules comprising codons that are preferred for expression by yeast cells. The synthetic molecules provided herein encode the same amino acid sequences as the wild-type HPV31 L1 gene.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a sequence alignment showing nucleotides that were altered in the partial (SEQ ID NO:2) and total rebuild (SEQ ID NO:3) 31 L1 genes (See EXAMPLE 2). The reference sequence is the 31 L1 wild-type sequence (SEQ ID NO:1; see EXAMPLE 1). Nucleotides in the 31 L1 partial and total rebuild sequences that are identical to the reference sequence are indicated with dots.

Altered nucleotides are indicated at their corresponding location. Nucleotide number is contained within the parentheses.

FIGURE 2 shows the 31 L1 total rebuild nucleotide (SEQ ID NO:3) and amino acid sequences (SEQ ID NO:4). The nucleotide number is indicated on the left.

FIGURE 3 summarizes the changes between the three HPV 31 L1 sequence constructs, which are listed on the left. The fourth column indicates the percent nucleotide identity between the indicated construct and the 31 L1 wild-type sequence and the fifth column indicates the amino acid identity. The last column indicates the number of nucleotides that were altered to yeast-preferred codon sequences and the region where the alterations were made.

FIGURE 4 shows a Northern blot probed specifically for HPV 31 L1 under high stringency (see EXAMPLE 4). Arrows on the left indicate the position of the HPV 31 L1 full length and truncated transcripts. Lanes labeled "31 wt" are from the same RNA preparation of yeast containing 31 L1 wild-type sequences. The lane labeled "16" contains RNA from HPV16, which is not recognized by the HPV 31 L1 probe because of the high stringency conditions. The lane labeled "Neg" is a yeast extract containing no L1 coding sequences. Lanes labeled "31 R" are from RNA of two separate isolated colonies expressing the 31 L1 partial rebuild sequence.

FIGURE 5 shows a portion of the data from two capture radioimmunoassay (RIA) experiments in counts per minute (cpm)/mg total protein (see EXAMPLE 7). Cpm obtained in the RIA are a relative indicator of HPV 31 L1 VLPs. The RIA data demonstrate increased 31 L1 VLP expression in yeast protein extracts from yeast-preferred codon rebuilt gene sequences.

FIGURE 6 shows a representative sample of the 31 L1 VLPs described herein, as visualized by transmission electron microscopy (see EXAMPLE 8). The bar represents 100 nm.

DETAILED DESCRIPTION OF THE INVENTION

The majority of cervical carcinomas are associated with infections of specific oncogenic types of human papillomavirus (HPV). The present invention relates to compositions and methods to elicit or enhance immunity to the protein products expressed by genes of oncogenic HPV types. Specifically, the present invention provides polynucleotides encoding HPV31 L1 and HPV31 virus-like particles (VLPs) and discloses use of said polynucleotides and VLPs in immunogenic compositions and vaccines for the prevention and/or treatment of HPV-associated cancer.

The wild-type HPV31 L1 nucleotide sequence has been reported (Goldsborough et al., *Virology* 171(1): 306-311 (1989); Genbank Accession # J04353). The present invention provides synthetic DNA molecules encoding the HPV31 L1 protein. The synthetic molecules of the present invention comprise a sequence of nucleotides, wherein some of the nucleotides have been altered so as to

eliminate transcription termination signals that are recognized by yeast. In alternative embodiments, the codons of the synthetic molecules are designed so as to use the codons preferred by a yeast cell for high-level expression. The synthetic molecules may be used as a source of HPV31 L1 protein, which may self-assemble into VLPs. Said VLPs may be used in a VLP-based vaccine to provide effective immunoprophylaxis against papillomavirus infection through neutralizing antibody and cell-mediated immunity. Such VLP-based vaccines are also useful for treatment of already established HPV infections.

Expression of HPV VLPs in yeast cells offers the advantages of being cost-effective and easily adapted to large-scale growth in fermenters. However, many HPV L1 proteins, including HPV31 L1 (see EXAMPLE 4), are expressed at low levels in yeast cells. It has been determined in accordance with the present invention that low level expression of HPV31 L1 is due to truncation of the mRNA transcript resulting from the presence of transcription termination signals that are recognized by yeast. By altering the HPV31 L1 DNA to eliminate any potential sequences resembling yeast transcription termination sites, it is possible to facilitate the transcription of full-length mRNA resulting in increased HPV31 L1 protein expression.

Accordingly, in some embodiments of this invention, alterations have been made to the HPV31 L1 DNA to eliminate any potential sequences resembling yeast transcription termination signals. These alterations allow expression of the full-length HPV31 transcript, as opposed to a truncated transcript (see EXAMPLE 4), improving expression yield.

As noted above, synthetic DNAs of the present invention comprise alterations from the wild-type HPV31 L1 sequence that were made to eliminate yeast-recognized transcription termination sites. One of skill in the art will recognize that additional DNA molecules can be constructed that encode the HPV31 L1 protein, but do not contain yeast transcription termination sites. Techniques for finding yeast transcription termination sequences are well known in the art. Transcription termination and 3' end formation of yeast mRNAs requires the presence of three signals: (1) an efficiency element such as TATATA or related sequences, which enhances the efficiency of positioning elements located downstream; (2) positioning element(s), which determine the location of the poly(A) site and (3) the polyadenylation site (usually Py(A)_n).

The scientific literature is replete with descriptions of sequences that encode yeast transcription termination signals. See, for example, Guo and Sherman, *Trends Biochem. Sci.* 21: 477-481 (1986); Guo and Sherman, *Mol. Cell. Biol.* 16(6): 2772-2776 (1996); Zaret et al, *Cell* 28:563-573 (1982); Henikoff et al, *Cell* 33:607-614 (1983); Thalenfeld et al, *J. Biol. Chem.* 258(23):14065-14068 (1983); Zaret et al, *J. Mol. Biol.* 176:107-135 (1984); Heidmann et al, *Mol. Cell Biol* 14:4633-4642 (1984); and Russo, *Yeast* 11:447-453 (1985). Therefore, one of skill in the art would have no difficulty determining which sequences to avoid in order to construct a synthetic HPV31 L1 gene that produces a full-length

mRNA transcript in accordance with the present invention. Additionally, assays and procedures to assess whether a yeast transcription termination sequence is present within the synthetic sequence are well established in the art, so that an ordinary skilled artisan would be able to determine if a constructed HPV31 L1 sequence comprises termination sequences that need to be eliminated.

5 As described above, the present invention relates to a nucleic acid molecule encoding HPV type 31 L1 protein, the nucleic acid molecule being free from internal transcription termination signals which are recognized by yeast. In exemplary embodiments of the invention, the synthetic nucleic acid molecules comprise a sequence of nucleotides as set forth in SEQ ID NO:2 or SEQ ID NO:3.

10 In alternative embodiments of the present invention, HPV31 L1 gene sequences are "optimized" for high level expression in a yeast cellular environment. Codon-optimized HPV31 L1 genes contemplated by the present invention include synthetic molecules encoding HPV31 L1 that are free from internal transcription termination signals which are recognized by yeast, further comprising at least one codon that is codon-optimized for high level expression in yeast cells.

A "triplet" codon of four possible nucleotide bases can exist in over 60 variant forms. Because these codons provide the message for only 20 different amino acids (as well as transcription initiation and termination), some amino acids can be coded for by more than one codon, a phenomenon known as codon redundancy. For reasons not completely understood, alternative codons are not uniformly present in the endogenous DNA of differing types of cells. Indeed, there appears to exist a variable natural hierarchy or "preference" for certain codons in certain types of cells. As one example, 20 the amino acid leucine is specified by any of six DNA codons including CTA, CTC, CTG, CTT, TTA, and TTG. Exhaustive analysis of genome codon frequencies for microorganisms has revealed endogenous DNA of *E. coli* most commonly contains the CTG leucine-specifying codon, while the DNA of yeasts and slime molds most commonly includes a TTA leucine-specifying codon. In view of this hierarchy, it is generally believed that the likelihood of obtaining high levels of expression of a leucine-rich polypeptide by an *E. coli* host will depend to some extent on the frequency of codon use. For 25 example, it is likely that a gene rich in TTA codons will be poorly expressed in *E. coli*, whereas a CTG rich gene will probably be highly expressed in this host. Similarly, a preferred codon for expression of a leucine-rich polypeptide in yeast host cells would be TTA.

30 The implications of codon preference phenomena on recombinant DNA techniques are manifest, and the phenomenon may serve to explain many prior failures to achieve high expression levels of exogenous genes in successfully transformed host organisms—a less "preferred" codon may be repeatedly present in the inserted gene and the host cell machinery for expression may not operate as efficiently. This phenomenon suggests that synthetic genes which have been designed to include a projected host cell's preferred codons provide an optimal form of foreign genetic material for practice of

recombinant DNA techniques. Thus, one aspect of this invention is an HPV31 L1 gene that is codon-optimized for expression in a yeast cell. In a preferred embodiment of this invention, it has been found that the use of alternative codons encoding the same protein sequence may remove the constraints on expression of HPV31 L1 proteins by yeast cells.

5 In accordance with this invention, HPV31 L1 gene segments were converted to sequences having identical translated sequences but with alternative codon usage as described by Sharp and Cowe (Synonymous Codon Usage in *Saccharomyces cerevisiae*. *Yeast* 7: 657-678 (1991)), which is hereby incorporated by reference. The methodology generally consists of identifying codons in the wild-type sequence that are not commonly associated with highly expressed yeast genes and replacing them
10 with optimal codons for high expression in yeast cells. The new gene sequence is then inspected for undesired sequences generated by these codon replacements (e.g., "ATTTA" sequences, inadvertent creation of intron splice recognition sites, unwanted restriction enzyme sites, etc.). Undesirable sequences are eliminated by substitution of the existing codons with different codons coding for the same amino acid. The synthetic gene segments are then tested for improved expression.

15 The methods described above were used to create synthetic gene segments for HPV31 L1, resulting in a gene comprising codons optimized for high level expression. While the above procedure provides a summary of our methodology for designing codon-optimized genes for use in HPV vaccines, it is understood by one skilled in the art that similar vaccine efficacy or increased expression of genes may be achieved by minor variations in the procedure or by minor variations in the sequence.

20 Accordingly, the present invention relates to a synthetic polynucleotide comprising a sequence of nucleotides encoding an HPV31 L1 protein, or a biologically active fragment or mutant form of an HPV31 L1 protein, the polynucleotide sequence comprising codons optimized for expression in a yeast host. Said mutant forms of the HPV31 L1 protein include, but are not limited to: conservative amino acid substitutions, amino-terminal truncations, carboxy-terminal truncations, deletions, or
25 additions. Any such biologically active fragment and/or mutant will encode either a protein or protein fragment which at least substantially mimics the immunological properties of the HPV31 L1 protein as set forth in SEQ ID NO:4. The synthetic polynucleotides of the present invention encode mRNA molecules that express a functional HPV31 L1 protein so as to be useful in the development of a therapeutic or prophylactic HPV vaccine.

30 One aspect of this invention is a codon-optimized nucleic acid molecule which encodes the HPV31 L1 protein as set forth in SEQ ID NO:4, said nucleic acid molecule comprising a sequence of nucleotides as set forth in SEQ ID NO:2.

Another aspect of this invention is a codon-optimized nucleic acid molecule which encodes the HPV31 L1 protein as set forth in SEQ ID NO:4, said nucleic acid molecule comprising a sequence of nucleotides as set forth in SEQ ID NO:3.

5 The present invention also relates to recombinant vectors and recombinant host cells, both prokaryotic and eukaryotic, which contain the nucleic acid molecules disclosed throughout this specification.

The synthetic HPV31 DNA or fragments thereof constructed through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred
10 into prokaryotic or eukaryotic host cells to produce recombinant HPV31 L1. Techniques for such manipulations are described in the art (Sambrook et al. Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, (1989); Current Protocols in Molecular Biology, Ausubel et al., Green Pub. Associates and Wiley-Interscience, New York (1988); Yeast Genetics: A Laboratory Course Manual, Rose et al., Cold Spring Harbor Laboratory, Cold Spring Harbor,
15 New York, (1990), which are hereby incorporated by reference in their entirety).

Thus, the present invention further relates to a process for expressing an HPV31 L1 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid encoding an HPV31 L1 protein into a yeast host cell; wherein the nucleic acid molecule is codon-optimized for optimal expression in the yeast host cell and; (b) culturing the yeast host cell under
20 conditions which allow expression of said HPV31 L1 protein.

The present invention also relates to a process for expressing an HPV31 L1 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid encoding an HPV31 L1 protein into a yeast host cell; wherein the nucleic acid molecule is free from internal transcription termination signals which are recognized by yeast and; (b) culturing the yeast host cell under conditions
25 which allow expression of said HPV31 L1 protein.

This invention further relates to a process for expressing an HPV31 L1 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid as set forth in SEQ ID NO:2 or SEQ ID NO:3 into a yeast host cell; and, (b) culturing the host cell under conditions which allow expression of said HPV31 L1 protein.

30 The synthetic genes of the present invention can be assembled into an expression cassette that comprises sequences designed to provide efficient expression of the HPV58 L1 protein in the host cell. The cassette preferably contains the synthetic gene, with related transcriptional and translations control sequences operatively linked to it, such as a promoter, and termination sequences. In a preferred embodiment, the promoter is the *S. cerevisiae GAL1* promoter, although those skilled in the art will

recognize that any of a number of other known yeast promoters such as the *GAL10*, *GAL7*, *ADH1*, *TDH3* or *PGK* promoters, or other eukaryotic gene promoters may be used. A preferred transcriptional terminator is the *S. cerevisiae ADH1* terminator, although other known transcriptional terminators may also be used. The combination of *GAL1* promoter – *ADH1* terminator is particularly preferred.

5 Another aspect of this invention is an HPV31 virus-like particle (VLP), methods of producing HPV31 VLPs, and methods of using HPV31 VLPs. VLPs can self-assemble when L1, the major capsid protein of human and animal papillomaviruses, is expressed in yeast, insect cells, mammalian cells or bacteria (for review, *see* Schiller and Roden, in *Papillomavirus Reviews: Current Research on Papillomaviruses*; Lacey, ed. Leeds, UK: Leeds Medical Information, pp 101-12 (1996)).
10 Morphologically indistinct HPV VLPs can also be produced by expressing a combination of the L1 and L2 capsid proteins. VLPs are composed of 72 pentamers of L1 in a T=7 icosahedral structure (Baker et al., *Biophys. J.* 60(6): 1445-56 (1991)).

VLPs are morphologically similar to authentic virions and are capable of inducing high titres of neutralizing antibodies upon administration into an animal. Immunization of rabbits (Breitburd et al., *J. Virol.* 69(6): 3959-63 (1995)) and dogs (Suzich et al., *Proc. Natl. Acad. Sci. USA* 92(25): 11553-57 (1995)) with VLPs was shown to both induce neutralizing antibodies and protect against experimental papillomavirus infection. However, because the VLPs do not contain the potentially oncogenic viral genome and can self-assemble from a single gene, they present a safe alternative to use of live virus in HPV vaccine development (for review, *see* Schiller and Hidesheim, *J. Clin. Virol.* 19: 67-74 (2000)).

20 Thus, the present invention relates to virus-like particles comprised of recombinant L1 protein or recombinant L1 + L2 proteins of HPV31.

In a preferred embodiment of the invention, the HPV31 VLPs are produced in yeast. In a further preferred embodiment, the yeast is selected from the group consisting of: *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces fragilis*, *Kluveromyces lactis*, and
25 *Schizosaccharomyces pombe*.

Another aspect of this invention is an HPV31 VLP, which comprises an HPV31 L1 protein produced by a HPV31 L1 gene that is free from internal transcription termination signals that are recognized by yeast.

Yet another aspect of this invention is an HPV31 VLP which comprises an HPV31 L1
30 protein produced by a codon-optimized HPV31 L1 gene. In a preferred embodiment of this aspect of the invention, the codon-optimized HPV31 L1 gene consists essentially of a sequence of nucleotides as set forth in SEQ ID NO:2 or SEQ ID NO:3.

Yet another aspect of this invention is a method of producing HPV31 VLPs, comprising:
(a) transforming yeast with a recombinant DNA molecule encoding HPV31 L1 protein or HPV31 L1 +

L2 proteins; (b) cultivating the transformed yeast under conditions that permit expression of the recombinant DNA molecule to produce the recombinant HPV31 protein; and (c) isolating the recombinant HPV31 protein to produce HPV31 VLPs.

In a preferred embodiment of this aspect of the invention, the yeast is transformed with a HPV31 L1 gene that is free from transcription termination signals that are recognized by yeast. In another preferred embodiment, the yeast is transformed with a codon-optimized HPV31 L1 gene to produce HPV31 VLPs. In a particularly preferred embodiment, the codon-optimized HPV31 L1 gene consists essentially of a sequence of nucleotides as set forth in SEQ ID NO:2 or SEQ ID NO:3.

This invention also provides a method for inducing an immune response in an animal comprising administering HPV31 virus-like particles to the animal. In a preferred embodiment, the HPV31 VLPs are produced by a gene that is free from internal transcription termination sequences that are recognized by yeast. In a further preferred embodiment, the HPV31 VLPs are produced by a codon-optimized gene.

Yet another aspect of this invention is a method of preventing or treating HPV-associated cervical cancer comprising administering to a mammal a vaccine comprising HPV31 VLPs. In a preferred embodiment of this aspect of the invention, the HPV31 VLPs are produced in yeast.

This invention also relates to a vaccine comprising HPV31 virus-like particles (VLPs).

In an alternative embodiment of this aspect of the invention, the vaccine further comprises VLPs of at least one additional HPV type. In a preferred embodiment, the at least one additional HPV type is selected from the group consisting of: HPV6, HPV11, HPV16, HPV18, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV55, HPV56, HPV58, HPV59, and HPV68.

In a preferred embodiment of this aspect of the invention, the vaccine further comprises HPV16 VLPs.

In another preferred embodiment of the invention, the vaccine further comprises HPV16 VLPs and HPV18 VLPs.

In yet another preferred embodiment of the invention, the vaccine further comprises HPV6 VLPs, HPV11 VLPs, HPV16 VLPs and HPV18 VLPs.

This invention also relates to pharmaceutical compositions comprising HPV 31 virus-like particles. Further, this invention relates to pharmaceutical compositions comprising HPV31 VLPs and VLPs of at least one additional HPV type. In a preferred embodiment, the at least one additional HPV type is selected from the group consisting of: HPV6, HPV11, HPV16, HPV18, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV55, HPV56, HPV58, HPV59, and HPV68.

Vaccine compositions of the present invention may be used alone at appropriate dosages defined by routine testing in order to obtain optimal inhibition of HPV31 infection while minimizing any

potential toxicity. In addition, co-administration or sequential administration of other agents may be desirable.

The amount of virus-like particles to be introduced into a vaccine recipient will depend on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 10 µg to 100 µg, and preferably about 20 µg to 60 µg of VLPs is administered directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations may be provided. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of administration with adjuvants such as alum or Merck alum adjuvant, concurrently with or subsequent to parenteral introduction of the vaccine of this invention is also advantageous.

All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing methodologies and materials that might be used in connection with the present invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Having described preferred embodiments of the invention with reference to the accompanying drawings, it is to be understood that the invention is not limited to those precise embodiments, and that various changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims.

The following examples illustrate, but do not limit the invention.

EXAMPLE 1

Determination of a representative HPV 31 L1 sequence

The HPV 31 L1 wild-type sequence has been described previously (Goldsborough et al., *Virology* 171(1): 306-311 (1989); Genbank Accession # J04353). It is not uncommon, however, to find minor sequence variations between DNAs obtained from clinical isolates. To isolate a representative HPV31 L1 wild-type sequence, DNA was isolated from three clinical samples previously shown to contain HPV 31 DNA. HPV 31 L1 sequences were amplified in a polymerase chain reaction (PCR) using *Taq* DNA polymerase and the following primers: HPV 31 L1 F 5' - CGT CGA CGT AAA CGT GTA TCA TAT TTT TTT ACA G - 3' (SEQ ID NO:5) and HPV 31 L1 B 5' - CAG ACA CAT GTA TTA CAT ACA CAA C - 3' (SEQ ID NO:6). The amplified products were electrophoresed on agarose gels

and visualized by ethidium bromide staining. The ~ 1500 bp L1 bands were excised and DNA purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany). The DNA was then ligated to the TA cloning vector, pCR-II (Invitrogen Corp., Carlsbad, CA), *E. coli* transformed, and plated on LB agar with ampicillin plus IPTG and X-gal for blue/white colony selection. The plates were inverted and
5 incubated for 16 hours at 37°C. White colonies were cultured in LB medium with ampicillin, shaking at 37°C for 16 hours, and minipreps were performed to extract the plasmid DNA.

To demonstrate the presence of the L1 gene in the plasmid, restriction endonuclease digestions were conducted and viewed by agarose gel electrophoresis and ethidium bromide staining. DNA sequencing was performed on plasmids containing cloned L1 from each of the three clinical
10 isolates. DNA and translated amino acid sequences were compared with one another and the Genbank HPV 31 L1 sequences. Sequence analysis of the three clinical isolates revealed that no sequence was identical to the Genbank sequence. The pCR-II-HPV 31L1/81 clone was chosen to be the representative 31L1 sequence and is referred to herein as the "31 L1 wild-type sequence" (SEQ ID NO:1, see FIGURE 1). The sequence chosen as 31 L1 wild-type contained one silent substitution at nucleotide 1266 and a
15 change from a C to a G at nucleotide 1295, altering the encoded amino acid from threonine to serine. The 31 L1 partial and total rebuilt genes (SEQ ID NOs: 2 and 3, respectively) also encode a serine at this location (see FIGURE 1). In all cases, the amino acid sequences are identical. Nucleotides were changed in the rebuilt constructs to encode amino acids using yeast-preferred codon sequences and to eliminate potential transcription termination signals (see EXAMPLE 2).

20 The 31 L1 wild-type sequence was amplified using the LS-101 5' - CTC AGA TCT CAC AAA ACA AAA TGT CTC TGT GGC GGC CTA GC - 3' (SEQ ID NO:7) and LS-102 5' - GAC AGA TCT TAC TTT TTA GTT TTT TTA CGT TTT GCT GG - 3' (SEQ ID NO:8) primers to add *Bgl*III extensions. PCR was performed using *Vent*TM DNA polymerase. The PCR product was visualized by ethidium bromide staining of an agarose gel. The ~ 1500 bp band was excised and DNA purified using
25 the QIAEX II gel extraction kit (Qiagen). The PCR product was then digested with *Bgl*III at 37 °C for 2 hours and purified using the QIA quick PCR purification kit. The *Bgl*III digested 31 L1 PCR product was ligated to *Bam*HI digested pGAL110 and DH5 *E. coli* were transformed. Colonies were screened by PCR for the HPV 31 L1 insert in the correct orientation. Sequence and orientation were confirmed by DNA sequencing. The selected clone was named pGAL110-HPV 31L1 #2.

30 Maxiprep DNA was then prepared and *Saccharomyces cerevisiae* were made competent and transformed. The yeast transformation was plated in Leu⁻ sorbitol top-agar on Leu⁻ sorbitol plates and incubated inverted for 3-5 days at 30°C. Colonies were picked and streaked for isolation on Leu⁻ sorbitol plates. To induce L1 transcription and protein expression, isolated colonies were subsequently

grown in 5 ml of 5 X Leu⁻ Ade⁻ sorbitol with 1.6% glucose and 4% galactose in rotating tube cultures at 30°C.

EXAMPLE 2

5 Yeast codon optimization

Yeast-preferred codons have been described (Sharp and Cowe, *Yeast* 7: 657-678 (1991)). Initially, the middle portion of HPV 31 L1, representing nucleotides 697-1249, was rebuilt utilizing yeast-preferred codons. The strategy employed to rebuild was to design long overlapping sense and antisense oligomers that span the region to be rebuilt, substituting nucleotides with yeast-preferred codon sequences while maintaining the same amino acid sequence. These oligomers were used in place of template DNA in the PCR reaction. Additional amplification primers were designed and used to amplify the rebuilt sequences from template oligomers with *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The optimal conditions for amplification were section-specific; however, most employed a program resembling the following: an initial denaturation step of 94°C for 1 minute, followed by 15-25 cycles of 95°C for 30 sec denature, 55°C for 30 sec anneal, 72°C for 3.5 minutes extension, followed by a 72°C for 10 minute final extension and 4°C hold.

PCR products were examined by agarose gel electrophoresis. Bands of the appropriate size were excised and the DNA was gel purified. The amplified fragments were then used as template to assemble the 552 nucleotide rebuilt HPV 31 middle L1 fragment. PCR was then used to amplify the wild-type nucleotides 1-725 (5'end) and 1221-1515 (3'end). A final PCR using the 5'end, the 3'end, and the rebuilt middle was performed to generate full-length 31 L1 partial rebuild, referred to herein as the "31 L1 partial rebuild".

The complete 31 L1 sequence was also rebuilt with yeast-preferred codons. This construct is referred to herein as the "31 L1 total rebuild". Nine long overlapping oligomers were used to generate yeast-preferred codon nucleotide sequences from 1-753 and four long overlapping oligomers were used to generate yeast-preferred codon nucleotide sequences from 1207-1515. After amplification and gel purification, these fragments, along with the middle rebuilt section described above (nucleotides 697-1249), were used together in a PCR reaction to generate the full length 31 L1 total rebuild sequence. This piece was generated with *Bam*HI extensions. The gel purified rebuilt 31L1 DNA was digested with *Bam*HI, ligated to *Bam*HI digested pGAL110 expression vector and transformed into *E. coli* DH5 cells. Colonies were screened by PCR for the HPV 31 L1 insert in the correct orientation. Sequence and orientation were confirmed by DNA sequencing.

Plasmid DNA was prepared. *S. cerevisiae* cells were made competent and transformed. The yeast were plated in Leu⁻ sorbitol top-agar on Leu⁻ sorbitol plates and incubated inverted for 3-5 days. Colonies were streaked for isolation on Leu⁻ sorbitol plates. Isolated colonies were subsequently grown in 5 ml of 5 X Leu⁻ Ade⁻ sorbitol with 1.6% glucose and 4% galactose in rotating tube cultures at 30°C to induce L1 transcription and protein expression. After 48-72 hours, culture volume equivalent to an OD600 = 10 was pelleted, supernate removed and the pellets frozen and stored -70°C.

EXAMPLE 3

10 RNA preparation

Cell pellets of transformed yeast, which were induced to express HPV 31 L1 by galactose induction, were thawed on ice and suspended in 1 ml of cold DEPC-treated water. Cells were pelleted by centrifugation and the resulting supernatant was removed. The cell pellet was then resuspended in 400 µl TES (10 mM Tris pH7.0, 10 mM EDTA and 0.5% SDS). An equal volume of AE buffer-saturated phenol (50 mM NaOAc and 10 mM EDTA) was added. The tube was vortexed for 10 seconds and heated to 65°C for 50 minutes with mixing every 10 minutes. The tube was then placed on ice for 5 minutes, followed by centrifugation at 4°C for 5 minutes. The supernatant was collected and transferred to a sterile tube. An additional 400 µl of phenol was added, the tube vortexed, placed on ice for 5 minutes and centrifuged. The supernatant was transferred to a sterile tube and 400 µl of chloroform added, mixed and centrifuged. The supernatant was again collected and transferred to a sterile tube and 40 µl 3 M Na Acetate pH 5.2 added in addition to 1 ml 100% EtOH. The tube was placed on dry ice for one hour, after which it was centrifuged at high speed to pellet the RNA. The RNA was washed one time with 70% EtOH and air-dried. The RNA was then suspended in 100 µl DEPC-treated water and heated to 65°C for 5 minutes to dissolve. Spectrophotometry was performed to determine the concentration of RNA in the sample using the assumption that an A260 reading of 1 = 40 µg/ml RNA when the A260/280 is 1.7-2.0.

EXAMPLE 4

Northern blot analysis

Initial analysis of yeast expressing 31 L1 wild-type suggested that the expression yield of HPV 31 L1 protein was considerably less than was expected. To determine if the low expression was occurring due to a problem at the transcription level versus the translation level, Northern blot analysis of the HPV 31 L1 transcript was performed. Northern blots were made from gels in which RNA from yeast expressing HPV16 L1 was run with RNA from yeast expressing HPV31 L1 on the same gel to compare transcript sizes.

A 1.2% agarose formaldehyde gel was cast. Ten micrograms of RNA was combined with denaturing buffer (final concentrations: 6% formaldehyde, 45% formamide and 0.9 x MOPS) and heated to 55°C for 15 minutes. A one-tenth volume of gel loading buffer was added and the sample loaded onto the gel. Electrophoresis was performed at 65 volts in 1 x MOPS buffer for ~ 5 hours. The gel was washed for 15 minutes in sterile water followed by two five minute washes in 10 x SSC. The RNA was transferred to a Hybond-N+ nylon membrane (Amersham Biosciences, Piscataway, NJ) by capillary action over 16 hours in 10 x SSC. The RNA was then fixed to the nylon membrane by cross-linking using the Amersham cross-linker set for 700 units of energy. After fixing, the nylon membrane was allowed to air dry. The membrane was placed in 30 ml Zetaprobe buffer at 55°C for 2 hours after which 32P-labeled probes were added and incubated for 16 hours at 53-65°C. The membrane was then washed 3 times in 5 X SSC at room temperature for 20 minutes, followed by 2 times in 0.4 x SSC for 20 minutes at room temperature and once at 60°C for 10 minutes. Probe DNA was generated by PCR using HPV 31 L1 sequence specific sense and antisense primers. The amplified DNA was labeled by treatment with polynucleotide kinase (PNK) and γ - 32P ATP at 37°C for 1 hour. The blot was wrapped in saran wrap and exposed to x-ray film for 16 hours. Upon film development, probe-hybridized RNA was detected as a black band on the autoradiograph.

Analysis of the Northern blot described above revealed that the majority of the full-length HPV 31 L1 wild-type transcripts were considerably smaller than full length (see FIGURE 4). However, the 31 L1 partial rebuild was designed not only to insert yeast-preferred codons in the middle of the gene, but also to eliminate any potential sequences resembling yeast transcription termination sites. Northern blot analysis clearly showed that upon rebuilding, the length of the 31 L1 gene transcript had significantly increased to a size corresponding with that of the full-length HPV 16 L1 transcript (not shown). Thus, premature transcription termination is likely to have accounted for a significant portion of the low expression yield from the 31 L1 wild-type construct.

EXAMPLE 5

HPV 31 L1 protein expression

Frozen yeast cell pellets of galactose induced cultures equivalent to OD600= 10 were thawed on ice and suspended in 300 μ l of PC buffer (100 mM Na₂HPO₄ and 0.5 M NaCl, pH 7.0) with 2mM PMSF. Acid-washed 0.5mm glass beads were added, ~ 0.5g/tube. The tubes were vortexed for 15 minutes at 4°C. 7.5 μ l of 20% TritonX100 was added and vortex repeated for 5 minutes at 4°C. The tubes were placed on ice for 15 minutes, then centrifuged for 15 minutes at 4°C. The supernate was transferred to a sterile microcentrifuge tube and stored at -70°C.

EXAMPLE 6

Western blot analysis

Total yeast protein extract from twenty to forty isolated yeast colonies for each HPV 31 L1 construct were analyzed by Western blot to confirm expression of HPV 31 L1 protein after galactose induction.

Ten micrograms of total yeast protein extract was combined with SDS-PAGE loading buffer and heated to 95°C for 10 minutes. The proteins were loaded onto an 8% SDS-PAGE gel and electrophoresed in Tris-Glycine buffer. After protein separation, the proteins were Western transferred from the gel to nitrocellulose and the blot was blocked in 10% non-fat dry milk in TTBS (Tris buffered saline with Tween -20) for 16 hours. The blot was washed three times in TTBS. Goat anti-trpE-HPV 16 L1 serum, a polyclonal serum that cross-reacts with HPV 31 L1, was applied at a 1:1000 dilution in TTBS for 1 hr at room temperature. The blot was washed three times in TTBS and anti-goat-HRP conjugated antibody was applied at a 1:2500 dilution in TTBS for 1 hr. The blot was again washed three times and ECL™ detection reagent was applied (Amersham Biosciences, Piscataway, NJ). Autoradiography was then performed. Proteins recognized by the antiserum were visualized by the detection reagent as dark bands on the autoradiograph.

In all cases, the HPV 31 L1 protein was detected as a distinct band on the autoradiograph corresponding to approximately 55 kD (data not shown). The HPV 16 L1 protein was included as a positive control on the gels.

EXAMPLE 7

Radioimmunoassay (RIA)

The yeast cells expressing HPV 31 L1 were grown by a variety of methods, including rotating tube cultures, shake flasks and fermenters. The yeast were lysed and protein extracts made to determine the amount of HPV 31 L1 virus-like particles (VLPs) produced per milligram of total protein. To demonstrate HPV 31 L1 VLP expression, a portion of each total yeast protein extract was analyzed by capture radioimmunoassay (RIA).

The RIA was performed using a detection monoclonal antibody, H31.A6, that is HPV type 31-specific and VLP conformational-specific. H31.A6 is specific for HPV type 31 L1 as it is found to bind intact HPV 31 L1 VLPs and does not recognize denatured HPV 31 VLPs. This mAb can be subsequently detected by a goat anti-mouse antibody radiolabeled with I125. Therefore, the counts per minute (cpm) values correspond to relative levels of HPV31 L1 VLP expression.

Polystyrene beads were coated with a goat anti-trpE-HPV31 L1 polyclonal serum diluted 1:1000 in PBS overnight. The beads were then washed with 5 volumes of sterile distilled water and air-dried. The antigen, total yeast protein extract from isolated yeast colonies, was then loaded onto the beads by dilution in PBS with 1% BSA, 0.1% Tween-20 and 0.1% Na Azide and incubated with rotation for one hour. After washing, the beads were distributed one per well in a 20-well polystyrene plate and incubated with H31.A6 mAb diluted 1:50,000 for 17-24 hours at room temperature. The beads were washed and I125 labeled goat anti-mouse IgG was added at an activity range of 23000-27000 cpm per 10 μ l. After 2 hours, the beads were washed and radioactive counts were recorded in cpm/ml. Background counts from blank wells were subtracted from the total cpm/ml, giving the RIA minus background value.

Two experiments were performed: in experiment 1, protein extracts from 31 L1 wild-type and 31 L1 partial rebuild were compared and in experiment 2, protein extracts from 31 L1 partial rebuild and 31 L1 total rebuild were compared (see FIGURE 5). Results indicate that 31 L1 partial rebuild VLP expression is 6.9 fold greater than 31 L1 wild-type. The 31 L1 total rebuild has a 1.7 fold increased expression over the 31 L1 partial rebuild. Therefore, the 31 L1 expression levels were increased > 7 fold by introducing yeast-preferred codon sequences and eliminating potential transcription termination signals.

EXAMPLE 8

Transmission electron microscopy

To demonstrate that the HPV 31 L1 protein was in fact self-assembling to form pentameric-L1 capsomers, which in turn self-assemble into virus-like particles, a partially purified 31 L1 total rebuild protein extract was subject to transmission electron microscopy (TEM). Yeast were grown under small scale fermentation and pelleted. The pellets were subjected to purification treatments. Pellet and clarified yeast extracts were analyzed by immunoblot to demonstrate L1 protein expression and retention through the purification procedure. Clarified yeast extracts were then subjected to centrifugation over a 45%-sucrose cushion and the resulting pellet suspended in buffer for TEM analysis (see FIGURE 6). Results indicated that the diameter of the spherical particles in this crude sample ranged from between 30 and 60 nm with some particles displaying a regular array of capsomers.

WHAT IS CLAIMED IS:

1. A nucleic acid molecule comprising a sequence of nucleotides that encodes an HPV31 L1 protein as set forth in SEQ ID NO:4, the nucleic acid sequence being codon-optimized for high level expression in a yeast cell.
2. A vector comprising the nucleic acid molecule of claim 1.
3. A host cell comprising the vector of claim 3.
4. The host cell of claim 3, wherein the host cell is selected from the group consisting of: *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe*.
5. The host cell of claim 4, wherein the host cell is *Saccharomyces cerevisiae*.
6. The nucleic acid molecule of claim 1, wherein the sequence of nucleotides comprises a sequence of nucleotides as set forth in SEQ ID NO:2.
7. A vector comprising the nucleic acid molecule of claim 6.
8. A host cell comprising the vector of claim 7.
9. The nucleic acid molecule of claim 1, wherein the sequence of nucleotides comprises a sequence of nucleotides as set forth in SEQ ID NO:3.
10. A vector comprising the nucleic acid molecule of claim 9.
11. A host cell comprising the vector of claim 10.
12. Virus-like particles (VLPs) comprised of recombinant L1 protein or recombinant L1 + L2 proteins of HPV31.

13. The VLPs of Claim 12 wherein the recombinant L1 protein or the recombinant L1 + L2 proteins are produced in yeast.

14. The VLPs of claim 13, wherein the recombinant L1 protein or recombinant L1 + L2 proteins are encoded by a codon-optimized HPV31 L1 nucleic acid molecule.

15. The VLPs of claim 14, wherein the codon-optimized nucleic acid molecule consists essentially of a sequence of nucleotides as set forth in SEQ ID NO:2 or SEQ ID NO:3.

16. A method of producing the VLPs of Claim 14, comprising:
(a) transforming yeast with a codon-optimized DNA molecule encoding HPV31 L1 protein or HPV31 L1 + L2 proteins;
(b) cultivating the transformed yeast under conditions that permit expression of the codon-optimized DNA molecule to produce a recombinant papillomavirus protein; and
(c) isolating the recombinant papillomavirus protein to produce the VLPs of Claim 14.

17. A vaccine comprising the VLPs of Claim 14.

18. Pharmaceutical compositions comprising the VLPs of claim 14.

19. A method of preventing HPV infection comprising administering the vaccine of Claim 17 to a mammal.

20. A method for inducing an immune response in an animal comprising administering the VLPs of Claim 14 to an animal.

21. The virus-like particles of Claim 14 wherein the yeast is selected from the group consisting of *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe*.

22. The virus-like particles of claim 21, wherein the yeast is *Saccharomyces cerevisiae*.

23. The vaccine of claim 17, further comprising VLPs of at least one additional HPV type.

24. The vaccine of claim 23, wherein the at least one additional HPV type is selected from the group consisting of: HPV6, HPV11, HPV16, HPV18, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV55, HPV56, HPV58, HPV59, and HPV68.

25. The vaccine of claim 24, wherein the at least one HPV type comprises HPV16.

26. The vaccine of claim 25, further comprising HPV18 VLPs.

27. The vaccine of claim 26, further comprising HPV6 VLPs and HPV11 VLPs.

28. A nucleic acid molecule comprising a sequence of nucleotides that encodes an HPV31 L1 protein, the nucleic acid molecule free from transcription termination signals that are recognized by yeast.

29. A vector comprising the nucleic acid molecule of claim 28.

30. A host cell comprising the vector of claim 29.

31. The host cell of claim 30, wherein the host cell is selected from the group consisting of: *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe*.

32. The host cell of claim 31, wherein the host cell is *Saccharomyces cerevisiae*.

33. The VLPs of claim 13, wherein the recombinant L1 protein or recombinant L1 + L2 proteins are encoded by a HPV31 L1 nucleic acid molecule that is free from transcription termination signals that are recognized by yeast.

34. A method of producing the VLPs of Claim 33, comprising:

- (a) transforming yeast with a DNA molecule encoding HPV31 L1 protein or HPV31 L1 + L2 proteins, the DNA molecule free from transcription termination sequences that are recognized by yeast;
- (b) cultivating the transformed yeast under conditions that permit expression of the DNA molecule to produce a recombinant papillomavirus protein; and
- (c) isolating the recombinant papillomavirus protein to produce the VLPs of Claim 33.

- 5
- 10 35. A vaccine comprising the VLPs of Claim 33.
36. Pharmaceutical compositions comprising the VLPs of claim 33.
- 15 37. A method of preventing HPV infection comprising administering the vaccine of Claim 35 to a mammal.
38. A method for inducing an immune response in an animal comprising administering the VLPs of Claim 33 to the animal.
- 20 39. The vaccine of claim 35, further comprising VLPs of at least one additional HPV type.
- 25 40. The vaccine of claim 39, wherein the at least one additional HPV type is selected from the group consisting of: HPV6, HPV11, HPV16, HPV18, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV55, HPV56, HPV58, HPV59, and HPV68.
- 30 41. The vaccine of claim 40, wherein the at least one HPV type comprises HPV16.
42. The vaccine of claim 41, further comprising HPV18 VLPs.
43. The vaccine of claim 42, further comprising HPV6 VLPs and HPV11 VLPs.

1/11

HPV 31 L1 nucleotide sequence alignment.

31 L1 wt	(1)	ATGTCTCTGTGGCGGCCTAGCGAGGCTACTGTCTACTTACCACCTGTCCC
31 L1 partial	(1)
31 L1 total	(1)T.....A.A..ATCT..A.....C.....G.....A.....
31 L1 wt	(51)	AGTGTCTAAAGTTGTAAGCACGGATGAATATGTAACACGAACCAACATAT
31 L1 partial	(51)
31 L1 total	(51)	...C.....G..C..CTCT..C..C.....C..C..CA.....C.
31 L1 wt	(101)	ATTATCACGCAGGCAGTGCTAGGCTGCTTACAGTAGGCCATCCATATTAT
31 L1 partial	(101)
31 L1 total	(101)	.C..C.....T..TTC.....AT..T.G..C..C..T..C.....C..C
31 L1 wt	(151)	TCCATACCTAAATCTGACAATCCTAAAAAATAGTTGTACCAAAGGTGTC
31 L1 partial	(151)
31 L1 total	(151)	..T..C..A..G.....C..A..G..G..C..C..C.....C..
31 L1 wt	(201)	AGGATTACAATATAGGGTATTTAGGGTTCGTTTACCAGATCCAAACAAAT
31 L1 partial	(201)
31 L1 total	(201)	T..T..G.....C..A..C..C..A..CA.A..G.....C.....G.
31 L1 wt	(251)	TTGGATTTCCTGATACATCTTTTTATAATCCTGAAACTCAACGCTTAGTT
31 L1 partial	(251)
31 L1 total	(251)	.C..T..C..A..C..C.....C..C..C..A.....C...A.A..G..C
31 L1 wt	(301)	TGGGCCTGTGTTGGTTTAGAGGTAGGTCGCGGGCAGCCATTAGGTGTAGG
31 L1 partial	(301)
31 L1 total	(301)T.....C.....G..A..C...A.A..T..A.....G.....C..
31 L1 wt	(351)	TATTAGTGGTCATCCATTATTAAATAAATTTGATGACACTGAAAACCTCTA
31 L1 partial	(351)
31 L1 total	(351)	...CTC.....C.....G..G..C..G..C..C.....C.....
31 L1 wt	(401)	ATAGATATGCCGGTGGTCCTGGCACTGATAATAGGGAATGTATATCAATG
31 L1 partial	(401)
31 L1 total	(401)	.C.....C..T.....A..T..C..C..C..A.....C..T...

FIG. 1A

2/11

31 L1 wt	(451)	GATTATAAACAAACACAACCTGTGTTTACTTGGTTGCAAACCACCTATTGG
31 L1 partial	(451)
31 L1 total	(451)	..C..C..G.....C...T.....GT.G.....T..G.....A..C..
31 L1 wt	(501)	AGAGCATTGGGGTAAAGGTAGTCCTTGTAGTAACAATGCTATTACCCCTG
31 L1 partial	(501)
31 L1 total	(501)	T..A..C.....G...TC...A...TC.....C.....C.....A.
31 L1 wt	(551)	GTGATTGTCCTCCATTAGAATTAATAAATTTCAGTTATACAAGATGGGGAT
31 L1 partial	(551)
31 L1 total	(551)C.....A.....G.....G..G..C..T..C..C.....C..T..C
31 L1 wt	(601)	ATGGTTGATACAGGCTTTGGAGCTATGGATTTTACTGCTTTACAAGACAC
31 L1 partial	(601)
31 L1 total	(601)C..C..C..T..C..T.....C..C..C.....G.....
31 L1 wt	(651)	TAAAAGTAATGTTCTTTGGACATTTGTAATTCTATTTGTAAATATCCAG
31 L1 partial	(651)
31 L1 total	(651)	C..GTC...C..C..A.....C.....C.....C.....G..C....
31 L1 wt	(701)	ATTATCTTAAATGGTTGCTGAGCCATATGGCGATACATTATTTTTTTAT
31 L1 partial	(701)C.....C..C..G..C..C..C
31 L1 total	(701)	.C..CT.G..G.....C.....A.....C.....C..C..G..C..C..C
31 L1 wt	(751)	TTACGTAGGGAACAAATGTTTGTAAGGCATTTTTTAATAGATCAGGCAC
31 L1 partial	(751)	..G.....A.....G.....C.....C..C..C..C.....C.....
31 L1 total	(751)	..G.....A.....G.....C.....C..C..C..C.....C.....
31 L1 wt	(801)	GGTTGGTGAATCGGTCCCTACTGACTTATATATTAAGGCTCCGGTTCAA
31 L1 partial	(801)	C..A.....T.....A..C...C.G..C..C..G.....C.
31 L1 total	(801)	C..A.....T.....A..C...C.G..C..C..G.....C.
31 L1 wt	(851)	CAGCTACTTTAGCTAACAGTACATACTTTCCTACACCTAGCGGCTCCATG
31 L1 partial	(851)	.C.....CC.G.....TCC..C.....C..A..T..ATCT.....
31 L1 total	(851)	.C.....CC.G.....TCC..C.....C..A..T..ATCT.....
31 L1 wt	(901)	GTTACTTCAGATGCACAAATTTTAATAAACCATATTGGATGCAACGTGC
31 L1 partial	(901)	..C..C..C..C..T..G..C..C..C..G.....C.....G.....
31 L1 total	(901)	..C..C..C..C..T..G..C..C..C..G.....C.....G.....

FIG. 1B

3/11

31 L1 wt	(951)	TCAGGGACACAATAATGGTATTTGTTGGGGCAATCAGTTATTTGTTACTG
31 L1 partial	(951)	A.....T.....C..C.....C.....T..C...C.G..C..G....
31 L1 total	(951)	A.....T.....C..C.....C.....T..C...C.G..C..G....
31 L1 wt	(1001)	TGGTAGATACCACACGTAGTACCAATATGTCTGTTTGTGCTGCAATTGCA
31 L1 partial	(1001)C.....G...TC.....C.....C.....C.....C..T
31 L1 total	(1001)C.....G...TC.....C.....C.....C.....C..T
31 L1 wt	(1051)	AACAGTGATACTACATTTAAAAGTAGTAATTTTAAAGAGTATTTAAGACA
31 L1 partial	(1051)	...TC...C.....C..C..GTCCTC...C..C..G.....CC.G.....
31 L1 total	(1051)	...TC...C.....C..C..GTCCTC...C..C..G.....CC.G.....
31 L1 wt	(1101)	TGGTGAGGAATTTGATTTACAATTTATATTTTCAGTTATGCAAAATAACAT
31 L1 partial	(1101)C...C.G.....C..C..C.....G.....G..C..CC
31 L1 total	(1101)C...C.G.....C..C..C.....G.....G..C..CC
31 L1 wt	(1151)	TATCTGCAGACATAATGACATATATTCACAGTATGAATCCTGCTATTTTG
31 L1 partial	(1151)	.G.....T.....C.....C..C..C.....C.....C..CC..
31 L1 total	(1151)	.G.....T.....C.....C..C..C.....C.....C..CC..
31 L1 wt	(1201)	GAAGATTGGAATTTTGGATTGACCACACCTCCCTCAGGTTCTTTGGAGGA
31 L1 partial	(1201)	..G..C.....C..C..TC.....T..A..T..C.....
31 L1 total	(1201)	..G..C.....C..C..TC.....T..A..T..C.....A..
31 L1 wt	(1251)	TACCTATAGGTTTGTAACTCACAGGCCATTACATGTCAAAAAGTGCCC
31 L1 partial	(1251)
31 L1 total	(1251)	C.....C..A..C..C.....T..A..T..C..C.....GTC...T.
31 L1 wt	(1301)	CCCAAAAGCCCAAGGAAGATCCATTTAAAGATTATGTATTTTGGGAGGTT
31 L1 partial	(1301)
31 L1 total	(1301)	.A.....A.....C.....C..G..C..C..C.....A..C
31 L1 wt	(1351)	AATTTAAAAGAAAAGTTTTCTGCAGATTTAGATCAGTTTCCACTGGGTCG
31 L1 partial	(1351)
31 L1 total	(1351)	..C..G..G.....C.....T..C..G..C..A..C...T....A.
31 L1 wt	(1401)	CAAATTTTATTACAGGCAGGATATAGGGCACGTCTAAATTTAAAGCAG
31 L1 partial	(1401)
31 L1 total	(1401)	A..G..C..G..G..A..T..T..C..A..TA..A..A..G..C..G..T.

FIG. 1C

4/11

31 L1 wt	(1451)	GTAAACGTAGTGCACCCTCAGCATCTACCACTACACCAGCAAAACGTAAA
31 L1 partial	(1451)
31 L1 total	(1451)GA.ATC...T..A..T..T.....C..C.....T..GA.A..G
31 L1 wt	(1501)	AAAACTAAAAAGTAA (SEQ ID NO:1)
31 L1 partial	(1501) (SEQ ID NO:2)
31 L1 total	(1501) (SEQ ID NO:3)

FIG.1D

5/11

HPV31 L1 total rebuild nucleotide and amino acid sequences.

M S L W R P S E A T V Y L P P V P
1 ATGTCTTTGT GGAGACCATC TGAAGCTACC GTCTACTTGC CACCAGTCCC

V S K V V S T D E Y V T R T N I Y
51 AGTCTCTAAG GTCGTCTCTA CCGACGAATA CGTCACCAGA ACCAACATCT

Y H A G S A R L L T V G H P Y Y
101 ACTACCACGC TGGTTCTGCT AGATTGTTGA CCGTCGGTCA CCCATACTAC

S I P K S D N P K K I V V P K V S
151 TCTATCCCAA AGTCTGACAA CCCAAAGAAG ATCGTCGTCC CAAAGGTCTC

G L Q Y R V F R V R L P D P N K F
201 TGGTTTGCAA TACAGAGTCT TCAGAGTCAG ATTGCCAGAC CCAAACAAGT

G F P D T S F Y N P E T Q R L V
251 TCGGTTTCCC AGACACCTCT TTCTACAACC CAGAAACCCA AAGATTGGTC

W A C V G L E V G R G Q P L G V G
301 TGGGCTTGTG TCGGTTTGGA AGTCGGTAGA GGTCAACCAT TGGGTGTCGG

I S G H P L L N K F D D T E N S N
351 TATCTCTGGT CACCCATTGT TGAACAAGTT CGACGACACC GAAAACTCTA

R Y A G G P G T D N R E C I S M
401 ACAGATACGC TGGTGGTCCA GGTACCGACA ACAGAGAATG TATCTCTATG

D Y K Q T Q L C L L G C K P P I G
451 GACTACAAGC AAACCCAATT GTGTTTGTTG GGTTGTAAGC CACCAATCGG

E H W G K G S P C S N N A I T P G
501 TGAACACTGG GGTAAGGGTT CTCCATGTTT TAACAACGCT ATCACCCCAG

D C P P L E L K N S V I Q D G D
551 GTGACTGTCC ACCATTGGAA TTGAAGAACT CTGTCATCCA AGACGGTGAC

FIG. 2A

6/11

M V D T G F G A M D F T A L Q D T
601 ATGGTCGACA CCGGTTTCGG TGCTATGGAC TTCACCGCTT TGCAAGACAC

K S N V P L D I C N S I C K Y P D
651 CAAGTCTAAC GTCCCATTGG ACATCTGTAA CTCTATCTGT AAGTACCCAG

Y L K M V A E P Y G D T L F F Y
701 ACTACTTGAA GATGGTCGCT GAACCATACG GCGACACCTT GTTCTTCTAC

L R R E Q M F V R H F F N R S G T
751 TTGCGTAGAG AACAGATGTT CGTAAGGCAC TTCTTCAACA GATCCGACAC

V G E S V P T D L Y I K G S G S T
801 CGTAGGTGAA TCTGTCCCAA CCGACCTGTA CATCAAGGGC TCCGGTTCCA

A T L A N S T Y F P T P S G S M
851 CCGCTACCCT GGCTAACTCC ACCTACTTCC CAACTCCATC TGGCTCCATG

V T S D A Q I F N K P Y W M Q R A
901 GTCACCTCCG ACGCTCAGAT CTTCAACAAG CCATACTGGA TGCAGCGTGC

Q G H N N G I C W G N Q L F V T V
951 ACAGGGTCAC AACAACGGTA TCTGTTGGGG TAACCAGCTG TTCGTGACTG

V D T T R S T N M S V C A A I A
1001 TGGTCGATAC CACGCGTTCT ACCAACATGT CTGTCTGTGC TGCAATCGCT

N S D T T F K S S N F K E Y L R H
1051 AACTCTGACA CTACCTTCAA GTCCTCTAAC TTCAAGGAGT ACCTGAGACA

G E E F D L Q F I F Q L C K I T L
1101 TGGTGAGGAA TTCGATCTGC AATTCATCTT CCAGTTGTGC AAGATCACCC

S A D I M T Y I H S M N P A I L
1151 TGTCTGCTGA CATCATGACC TACATCCACA GTATGAACCC TGCCATCCTG

E D W N F G L T T P P S G S L E D
1201 GAGGACTGGA ACTTCGGTCT GACCACTCCA CCTTCCGGTT CTTTGGAAGA

FIG.2B

7/11

1251 T Y R F V T S Q A I T C Q K S A P
CACCTACAGA TTCGTCACCT CTCAAGCTAT CACCTGTCAA AAGTCTGCTC

1301 Q K P K E D P F K D Y V F W E V
CACAAAAGCC AAAGGAAGAC CCATTCAAGG ACTACGTCTT CTGGGAAGTC

1351 N L K E K F S A D L D Q F P L G R
AACTTGAAGG AAAAGTTCTC TGCTGACTTG GACCAATTCC CATTGGGCTAG

1401 K F L L Q A G Y R A R P K F K A G
AAAGTTCTTG TTGCAAGCTG GTTACAGAGC TAGACCAAAG TTCAAGGCTG

1451 K R S A P S A S T T T P A K R K
GTAAGAGATC TGCTCCATCT GCTTCTACCA CCACCCCAGC TAAGAGAAAG

1501 K T K K * (SEQ ID NO:4)
AAGACCAAGA AGTAA (SEQ ID NO:3)

FIG.2C

8/11

SUMMARY OF HPV 31 SEQUENCES.

L1 CONSTRUCT	NUCLEOTIDES	AMINO ACIDS	NUCLEOTIDE IDENTITY	AMINO ACID IDENTITY	COMMENTS
HPV 31 LA WILD-TYPE	1515	504			
HPV 31 L1 PARTIAL REBUILD	1515	504	92%	100%	121 CHANGES BETWEEN nt 697-1249
HPV 31 L1 TOTAL REBUILD	1515	504	75%	100%	376 CHANGES BETWEEN nt 1-1515

FIG.3

9/11

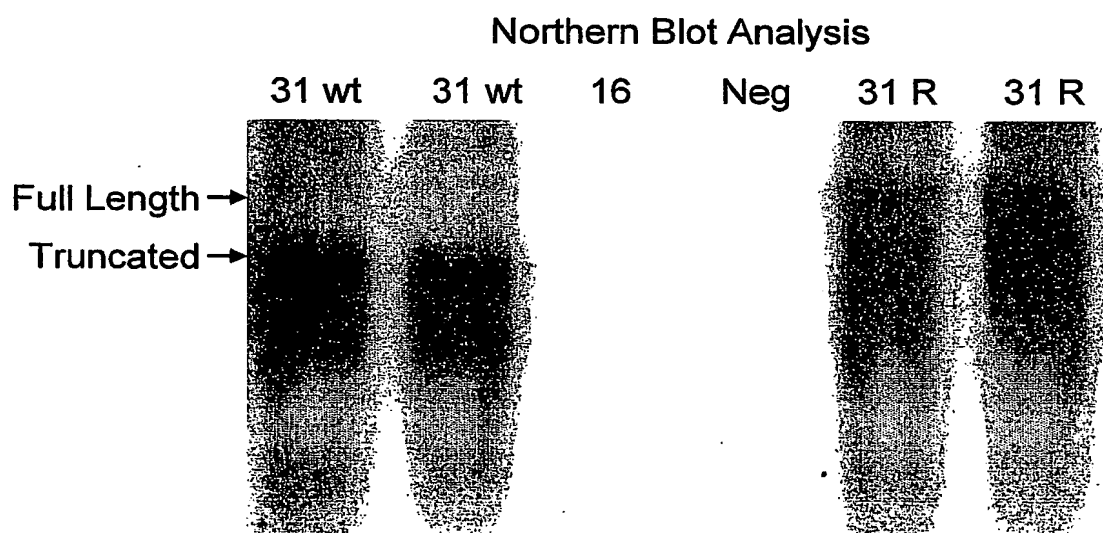


FIG.4

10/11

RESULTS FROM RADIOIMMUNOASSAY

EXP.	L1 CONSTRUCT	PROTEIN CONCENTRATION (mg/ml)	RIA MINUS BACKGROUND (cpm/ml)	31 L1 VLP*/ mg protein (cpm/mg)	AVERAGE (cpm/mg)	RATIO (Ave/Ave)
1	31 WILD-TYPE	1.64	414	252		
		1.62	987	609	460	1
		1.82	904	497		(31 wt/31 wt)
		1.76	844	480		
1	31 PARTIAL REBUILD	1.67	5061	3031	3158	6.9
		1.55	5091	3285		(31 PARTIAL/31 wt)
2	31 PARTIAL REBUILD	2.27	3901	1719		
		2.27	4081	1798	2095	1
		2.38	7135	2998		(31 PARTIAL/31 PARTIAL)
		2.38	4428	1861		
2	31 TOTAL REBUILD	2.07	6628	3202	3645	1
		2.07	8461	4087		(31 TOTAL/31 PARTIAL)

FIG. 5

11/11

Transmission Electron Microscopy

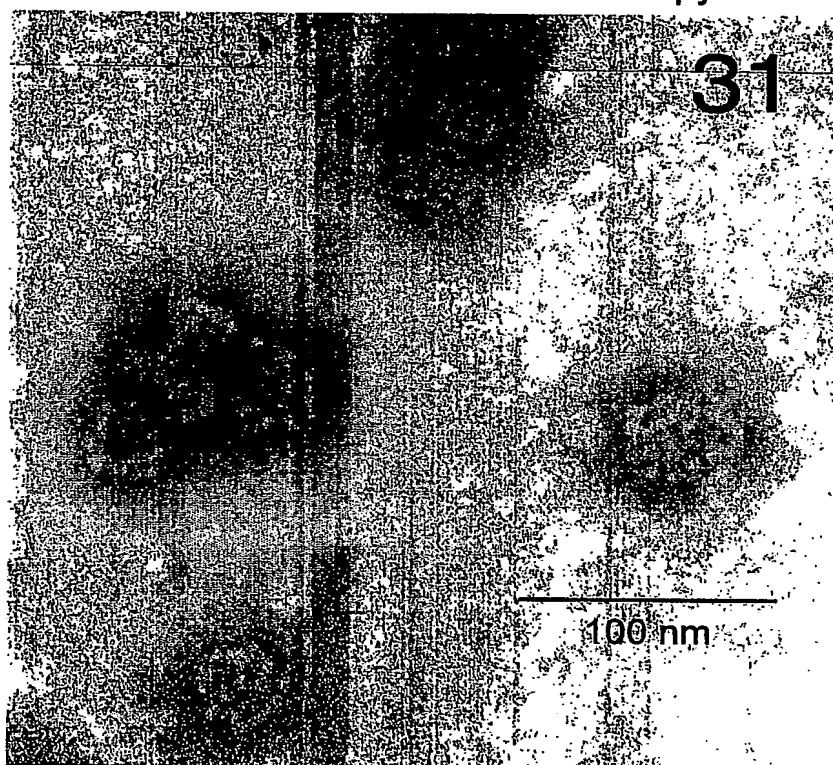


FIG.6

SEQUENCE LISTING

<110> Merck & Co., Inc.
Jansen, Kathrin U.
Schultz, Loren D.
Neeper, Michael P.
Markus, Henry Z.

<120> OPTIMIZED EXPRESSION OF HPV 31 L1 IN
YEAST

<130> 21188-PCT

<150> 60/457,172

<151> 2003-03-24

<160> 8

<170> FastSEQ for Windows Version 4.0

<210> 1

<211> 1515

<212> DNA

<213> HPV31 L1 wild-type

<400> 1

```

atgtctctgt ggcggcctag cgaggctact gtctacttac cacctgtccc agtgtctaaa 60
gttgtaagca cggatgaata tgtaacacga accaaccatat attatcacgc aggcagtgct 120
aggctgctta cagtaggcca tccatattat tccataccta aatctgacaa tcctaaaaaa 180
atagttgtac caaagggtgc aggattacaa tataggggtat ttaggggttcg ttaccagat 240
ccaaacaaat ttggatttcc tgatacatct ttttataatc ctgaaactca acgcttagtt 300
tgggcctgtg ttggtttaga ggtaggtcgc gggcagccat taggtgtagg tattagtggt 360
catccattat taaataaatt tgatgacact gaaaactcta atagatatgc cgggtggctct 420
ggcactgata ataggggaatg tatatcaatg gattataaac aaacacaact gtgtttactt 480
ggttgcaaac caccatttgg agagcattgg ggtaaaggta gtccttgtag taacaatgct 540
attacccttg gtgattgtcc tccattagaa ttaaaaaatt cagttataca agatggggat 600
atgggtgata caggctttgg agctatggat tttactgctt tacaagacac taaaagtaat 660
gttccttttg acatttgtaa ttctatttgt aaatatccag attatcttaa aatgggttgct 720
gagccatatg gcgatacatt atttttttat ttacgtaggg aacaaatggt tgtaaggcat 780
ttttttaata gatcaggcac gggtggtgaa tcgggtcccta ctgacttata tattaaaggc 840
tccggttcaa cagctacttt agctaacagt acatactttc ctacacctag cggctccatg 900
gttacttcag atgcacaaat ttttaataaa ccatattgga tgcaacgtgc tcagggacac 960
aataatggta tttgttgggg caatcagtta tttgttactg tggtagatac cacacgtagt 1020
accaatatgt ctgtttgtgc tgcaattgca aacagtgata ctacatttaa aagtagtaat 1080
tttaaagagt atttaagaca tgggtaggaa tttgatttac aatttatatt tcagttatgc 1140
aaaataacat tatctgcaga cataatgaca tatattcaca gtatgaatcc tgctattttg 1200
gaagattgga attttggatt gaccacacct ccctcaggtt ctttggagga tacctatagg 1260
tttgaacct cacaggccat tacatgtcaa aaaagtgtcc cccaaaagcc caaggaagat 1320
ccatttaaag attatgtatt ttgggaggtt aattttaaag aaaagttttc tgcagattta 1380
gatcagtttc cactgggtcg caaattttta ttacaggcag gatatagggc acgtcctaaa 1440
tttaaagcag gtaaacgtag tgcacctca gcatctacca ctacaccagc aaaacgtaaa 1500
aaaactaaaa agtaa 1515

```

<210> 2

<211> 1515

<212> DNA

<213> Artificial Sequence

<220>

<223> 31 partial rebuild

<400> 2

atgtctctgt	ggcggcctag	cgaggctact	gtctacttac	cacctgtccc	agtgtctaaa	60
ggtgtaagca	cggatgaata	tgtaacacga	accaacatat	attatcacgc	aggcagtgtc	120
aggctgctta	cagtaggcca	tccatattat	tccataccta	aatctgacaa	tcctaaaaaa	180
atagttgtac	caaagggtgc	aggattacaa	tatagggtat	ttaggggtcg	tttaccagat	240
ccaaacaaat	ttggatttcc	tgatacatct	ttttataatc	ctgaaactca	acgcttagtt	300
tgggcctgtg	ttggtttaga	ggtaggtcgc	gggcagccat	taggtgtagg	tattagtgtt	360
catccattat	taaataaatt	tgatgacact	gaaaactcta	atagatatgc	cgggtgtcct	420
ggcactgata	atagggaatg	tatatcaatg	gattataaac	aaacacaaact	gtgtttactt	480
ggttgcaaac	cacctattgg	agagcattgg	ggtaaaggta	gtccttgtag	taacaatgct	540
attacccttg	gtgattgtcc	tccattagaa	ttaaaaaatt	cagttataca	agatggggat	600
atggttgata	caggcttttg	agctatggat	tttactgtct	tacaagacac	taaaagtaat	660
gttccttttg	acatttgtaa	ttctatttgt	aaatatccag	attatcttaa	aatgggtgtc	720
gagccatacg	gcgacacctt	gttcttctat	ttgcgtagag	aacagatgtt	cgtaaggcac	780
ttcttcaaca	gatccggcac	cgtaggtgaa	tctgtcccaa	ccgacctgta	catcaagggc	840
tccgggtcca	ccgctaccct	ggctaactcc	acctacttcc	caactccatc	tggctccatg	900
gtcacctccg	acgctcagat	cttcaacaag	ccatactgga	tgcagcgtgc	acagggtcac	960
aacaacggta	tctgttgggg	taaccagctg	ttcgtgactg	tggctgatac	cacgcgttct	1020
accaacatgt	ctgtctgtgc	tgcaatcgct	aactctgaca	ctaccttcaa	gtcctctaac	1080
ttcaaggagt	acctgagaca	tggtgaggaa	ttcgatctgc	aattcatctt	ccagttgtgc	1140
aagatcaccc	tgtctgctga	catcatgacc	tacatccaca	gtatgaaccc	tgccatcctg	1200
gaggactgga	acttcgggtc	gaccactcca	ccttcgggtt	ctttggagga	tacctatagg	1260
tttgtaacct	cacaggccat	tacatgtcaa	aaaagtgtcc	cccaaaagcc	caaggaagat	1320
ccatttaaag	attatgtatt	ttgggaggtt	aattttaaag	aaaagttttc	tgcagattta	1380
gatcagtttc	catgggtcgc	caaattttta	ttacaggcag	gatatagggc	acgtcctaaa	1440
tttaaagcag	gtaaacgtag	tgcacctca	gcacttacca	ctacaccagc	aaaacgtaaa	1500
aaaactaaaa	agtaa					1515

<210> 3

<211> 1515

<212> DNA

<213> Artificial Sequence

<220>

<223> 31 total rebuild

<400> 3

atgtctttgt	ggagaccatc	tgaagctacc	gtctacttgc	caccagtccc	agtctctaag	60
gtcgtctcta	ccgacgaata	cgtcaccaga	accaacatct	actaccacgc	tggttctgtc	120
agattgttga	ccgtcgggtca	cccatactac	tctatcccaa	agtctgacaa	cccaaagaag	180
atcgtcgtcc	caaagggtctc	tggtttgcaa	tacagagtct	tcagagtccg	attgccagac	240
ccaaacaagt	tccggtttccc	agacacctct	ttctacaacc	cagaaaccca	aagattgggtc	300
tgggcttgtg	tccggtttgga	agtccggtaga	ggccaaccat	tgggtgtcgg	tatctctggt	360
caccatttgt	tgaacaagtt	cgacgacacc	gaaaactcta	acagatacgc	tgggtgtcca	420
ggtaccgaca	acagagaatg	tatctctatg	gactacaagc	aaacccaatt	gtgtttgttg	480
ggttgtaagc	caccaatcgg	tgaacactgg	ggtaagggtt	ctccatgttc	taacaacgct	540
atcaccccgag	gtgactgtcc	accattggaa	ttgaagaact	ctgtcatcca	agacgggtgac	600
atggctgaca	ccggttttcgg	tgctatggac	ttcaccgctt	tgcaagacac	caagtctaac	660
gtcccatttg	acatctgtaa	ctctatctgt	aagtacccag	actacttgaa	gatggctcgt	720
gaaccatacg	gcgacacctt	gttcttctac	ttgcgtagag	aacagatgtt	cgtaaggcac	780
ttcttcaaca	gatccggcac	cgtaggtgaa	tctgtcccaa	ccgacctgta	catcaagggc	840
tccggttcca	ccgctaccct	ggctaactcc	acctacttcc	caactccatc	tggctccatg	900
gtcacctccg	acgctcagat	cttcaacaag	ccatactgga	tgcagcgtgc	acagggtcac	960
aacaacggta	tctgttgggg	taaccagctg	ttcgtgactg	tggctgatac	cacgcgttct	1020
accaacatgt	ctgtctgtgc	tgcaatcgct	aactctgaca	ctaccttcaa	gtcctctaac	1080
ttcaaggagt	acctgagaca	tggtgaggaa	ttcgatctgc	aattcatctt	ccagttgtgc	1140
aagatcaccc	tgtctgctga	catcatgacc	tacatccaca	gtatgaaccc	tgccatcctg	1200
gaggactgga	acttcgggtc	gaccactcca	ccttcgggtt	ctttggaaga	cacctacaga	1260
ttcgtcacct	ctcaagctat	cacctgtcaa	aagtctgtct	cacaaaagcc	aaaggaagac	1320
ccattcaagg	actacgtctt	ctgggaagtc	aacttgaagg	aaaagttctc	tgctgacttg	1380
gaccaattcc	cattgggtag	aaagttcttg	ttgcaagctg	gttacagagc	tagaccaaaag	1440
ttcaaggctg	gtaagagatc	tgtccatct	gcttctacca	ccaccccgagc	taagagaaaag	1500

aagaccaaga agtaa

1515

<210> 4
 <211> 504
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> HPV 31 L1

<400> 4
 Met Ser Leu Trp Arg Pro Ser Glu Ala Thr Val Tyr Leu Pro Pro Val
 1 5 10 15
 Pro Val Ser Lys Val Val Ser Thr Asp Glu Tyr Val Thr Arg Thr Asn
 20 25 30
 Ile Tyr Tyr His Ala Gly Ser Ala Arg Leu Leu Thr Val Gly His Pro
 35 40 45
 Tyr Tyr Ser Ile Pro Lys Ser Asp Asn Pro Lys Lys Ile Val Val Pro
 50 55 60
 Lys Val Ser Gly Leu Gln Tyr Arg Val Phe Arg Val Arg Leu Pro Asp
 65 70 75 80
 Pro Asn Lys Phe Gly Phe Pro Asp Thr Ser Phe Tyr Asn Pro Glu Thr
 85 90 95
 Gln Arg Leu Val Trp Ala Cys Val Gly Leu Glu Val Gly Arg Gly Gln
 100 105 110
 Pro Leu Gly Val Gly Ile Ser Gly His Pro Leu Leu Asn Lys Phe Asp
 115 120 125
 Asp Thr Glu Asn Ser Asn Arg Tyr Ala Gly Gly Pro Gly Thr Asp Asn
 130 135 140
 Arg Glu Cys Ile Ser Met Asp Tyr Lys Gln Thr Gln Leu Cys Leu Leu
 145 150 155 160
 Gly Cys Lys Pro Pro Ile Gly Glu His Trp Gly Lys Gly Ser Pro Cys
 165 170 175
 Ser Asn Asn Ala Ile Thr Pro Gly Asp Cys Pro Pro Leu Glu Leu Lys
 180 185 190
 Asn Ser Val Ile Gln Asp Gly Asp Met Val Asp Thr Gly Phe Gly Ala
 195 200 205
 Met Asp Phe Thr Ala Leu Gln Asp Thr Lys Ser Asn Val Pro Leu Asp
 210 215 220
 Ile Cys Asn Ser Ile Cys Lys Tyr Pro Asp Tyr Leu Lys Met Val Ala
 225 230 235 240
 Glu Pro Tyr Gly Asp Thr Leu Phe Phe Tyr Leu Arg Arg Glu Gln Met
 245 250 255
 Phe Val Arg His Phe Phe Asn Arg Ser Gly Thr Val Gly Glu Ser Val
 260 265 270
 Pro Thr Asp Leu Tyr Ile Lys Gly Ser Gly Ser Thr Ala Thr Leu Ala
 275 280 285
 Asn Ser Thr Tyr Phe Pro Thr Pro Ser Gly Ser Met Val Thr Ser Asp
 290 295 300
 Ala Gln Ile Phe Asn Lys Pro Tyr Trp Met Gln Arg Ala Gln Gly His
 305 310 315 320
 Asn Asn Gly Ile Cys Trp Gly Asn Gln Leu Phe Val Thr Val Val Asp
 325 330 335
 Thr Thr Arg Ser Thr Asn Met Ser Val Cys Ala Ala Ile Ala Asn Ser
 340 345 350
 Asp Thr Thr Phe Lys Ser Ser Asn Phe Lys Glu Tyr Leu Arg His Gly
 355 360 365
 Glu Glu Phe Asp Leu Gln Phe Ile Phe Gln Leu Cys Lys Ile Thr Leu
 370 375 380
 Ser Ala Asp Ile Met Thr Tyr Ile His Ser Met Asn Pro Ala Ile Leu
 385 390 395 400
 Glu Asp Trp Asn Phe Gly Leu Thr Thr Pro Pro Ser Gly Ser Leu Glu

Asp	Thr	Tyr	Arg	405	Phe	Val	Thr	Ser	Gln	410	Ala	Ile	Thr	Cys	Gln	415	Lys	Ser
			420							425						430		
Ala	Pro	Gln	Lys	Pro	Lys	Glu	Asp	Pro	Phe	Lys	Asp	Tyr	Val	Phe	Trp			
		435					440						445					
Glu	Val	Asn	Leu	Lys	Glu	Lys	Phe	Ser	Ala	Asp	Leu	Asp	Gln	Phe	Pro			
		450				455						460						
Leu	Gly	Arg	Lys	Phe	Leu	Leu	Gln	Ala	Gly	Tyr	Arg	Ala	Arg	Pro	Lys			
465					470					475					480			
Phe	Lys	Ala	Gly	Lys	Arg	Ser	Ala	Pro	Ser	Ala	Ser	Thr	Thr	Thr	Pro			
				485					490						495			
Ala	Lys	Arg	Lys	Lys	Thr	Lys	Lys											
			500															

<210> 5
 <211> 34
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> PCR Primer

<400> 5
 cgctgcacgta aacgtgtatc atattttttt acag 34

<210> 6
 <211> 25
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> PCR Primer

<400> 6
 cagacacatg tattacatac acaac 25

<210> 7
 <211> 41
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> PCR Primer

<400> 7
 ctcagatctc acaaaacaaa atgtctctgt ggcggcctag c 41

<210> 8
 <211> 38
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> PCR Primer

<400> 8
 gacagatctt acttttttagt ttttttacgt tttgctgg 38

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
7 October 2004 (07.10.2004)

PCT

(10) International Publication Number
WO 2004/084831 A3

- (51) International Patent Classification⁷: **C12P 21/06**
- (21) International Application Number:
PCT/US2004/008677
- (22) International Filing Date: 19 March 2004 (19.03.2004)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/457,172 24 March 2003 (24.03.2003) US
- (71) Applicant (for all designated States except US): **MERCK & CO. INC.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **JANSEN, Kathrin, U.** [DE/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **SCHULTZ, Loren, D.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **NEEPER, Michael, P.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **MARKUS, Henry, Z.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).
- (74) Common Representative: **MERCK & CO. INC.**; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
- with international search report
 - before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- (88) Date of publication of the international search report:
11 November 2004
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: OPTIMIZED EXPRESSION OF HPV 31 L1 IN YEAST

(57) Abstract: Synthetic DNA molecules encoding the HPV31 L1 protein are provided. Specifically, the present invention provides polynucleotides encoding HPV31 L1 protein, wherein said polynucleotides are free from internal transcription termination signals that are recognized by yeast. Also provided are synthetic polynucleotides encoding HPV31 L1 wherein the polynucleotides have been codon-optimized for high level expression in a yeast cell. The synthetic molecules may be used to produce HPV31 virus-like particles (VLPs), and to produce vaccines and pharmaceutical compositions comprising the HPV31 VLPs. The vaccines of the present invention provide effective immunoprophylaxis against papillomavirus infection through neutralizing antibody and cell-mediated immunity.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/08677

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 21/06
US CL : 435/69.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/69.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 01/14416 A2 (MERCK & CO., INC) 01 March 2001, see the abstract.	1-43
A	US 5,821,087 A (LOWE et al) 13 October 1998 (13.10.1998), abstract.	1-43
A	WO 00/09157 A1 (MERCK & Co. INC.) 24 February 2000 (24.02.2000), abstract.	1-43

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

28 July 2004 (28.07.2004)

Date of mailing of the international search report

24 SEP 2004

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Facsimile No. (703) 305-3230

Authorized officer

Valerie Bell-Harris
A. R. Sahni

Telephone No. (571) 272-1600

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US04/08677

Continuation of B. FIELDS SEARCHED Item 3:

WEST, DERWENT, EPA, JPA, CAPLUS, BIOSIS, MEDLINE, NPL

search terms: Papilloma virus, HPV?, codon optimized, yeast, vector, vaccine, CTL

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☒ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.